

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Shaker A. Mousa

Group Art Unit: 1623

Application No.: 10/667,216

Examiner: Issac, Roy P.

Filing Date: 09/19/2003

Docket No.: **MOUSA-4618**

Title: **OXIDIZED HEPARIN FRACTIONS AND THEIR USE IN INHIBITING
ANGIOGENESIS**

APPENDIX A

Copy Of:

Lars Lundin et al.,

Selectively Desulfated Heparin Inhibits Fibroblast

Growth Factor-induced Mitogenicity and Angiogenesis,

Journal of Biological Chemistry, Vol. 275, No. 32

(August 11, 2000)

Selectively Desulfated Heparin Inhibits Fibroblast Growth Factor-induced Mitogenicity and Angiogenesis*

Received for publication, November 5, 1999, and in revised form, April 14, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M908930199

Lars Lundin‡, Helena Larsson‡, Johan Kreuger§, Shigeru Kanda¶, Ulf Lindahl§, Markku Salmivirta§, and Lena Claesson-Welsh‡¶

From the ‡Department of Genetics and Pathology, Rudbeck Laboratory, S-751 85 Uppsala, Sweden, §Department of Medical Biochemistry and Microbiology, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden, and ¶Nagasaki University School of Medicine, Nagasaki 852-8501, Japan

Fibroblast growth factors (FGFs) are known to induce formation of new blood vessels, angiogenesis. We show that FGF-induced angiogenesis can be modulated using selectively desulfated heparin. Chinese hamster ovary cells (CHO677) deficient in heparan sulfate biosynthesis were employed to assess the function of heparin/heparan sulfate in FGF receptor-1 (FGFR-1) signal transduction and biological responses. In the presence of FGF-2, FGFR-1 kinase and subsequent mitogen-activated protein kinase Erk2 activities were augmented in a dose-dependent manner, whereas high concentrations of heparin resulted in decreased activity. The length of the heparin oligomer, minimally an 8/10-mer, was critical for the ability to enhance FGFR-1 kinase activity. The *N*- and 2-*O*-sulfate groups of heparin were essential for binding to FGF-2, whereas stimulation of FGFR-1 and Erk2 kinases by FGF-2 also required the presence of 6-*O*-sulfate groups. Sulfation at 2-*O*- and 6-*O*-positions was moreover a prerequisite for binding of heparin to a lysine-rich peptide corresponding to amino acids 160–177 in the extracellular domain of FGFR-1. Selectively 6-*O*-desulfated heparin, which binds to FGF-2 but fails to bind the receptor, decreased FGF-2-induced proliferation of CHO677 cells, presumably by displacing intact heparin. Furthermore, FGF-2-induced angiogenesis in chick embryos was inhibited by 6-*O*-desulfated heparin. Thus, formation of a ternary complex of FGF-2, heparin, and FGFR-1 appears critical for the activation of FGFR-1 kinase and downstream signal transduction. Preventing complex formation by modified heparin preparations may allow regulation of FGF-2 functions, such as induction of angiogenesis.

Fibroblast growth factors (FGFs)¹ constitute a family of about 20 structurally related polypeptides, which regulate cell growth, motility, and differentiation. FGFs exert their effects by binding with high affinity to four distinct but highly related

receptor tyrosine kinases (FGF receptor-1, -2, -3, and -4) (1). Binding of FGF to FGF receptors leads to receptor dimerization and autophosphorylation of the intracellular receptor domain (2). FGF receptor autophosphorylation allows binding of signal transduction molecules, resulting in activation of signal transduction pathways and eventually in cellular responses such as proliferation, migration, and differentiation. The Ras pathway is known to be important in several biological responses to FGF. Key components in this pathway are the mitogen-activated protein kinases Erk1 and Erk2, which are activated and translocated to the nucleus where they phosphorylate and thereby regulate the activities of certain early gene transcription factors, such as Myc and Fos (1). FGF has also been shown to be translocated to the nucleus and induce DNA synthesis independently of FGF receptors (3).

It is well established that FGFs bind to heparan sulfate (HS)/heparin, and there are many examples of the biological significance of such interactions. Binding of FGF to HS on the cell surface and in the extracellular matrix protects FGF from degradation (4). The HS-immobilized FGF can be released by heparanases and may act *e.g.* in injury repair processes (5). HS is furthermore directly implicated in the biological action of FGF. Cells deficient in endogenous HS are not responsive to FGF, but the responsiveness can be restored by the addition of exogenous heparin (6). HS and heparin are structurally related, sulfated glycosaminoglycans composed of alternating hexuronic acid and glucosamine units. The biological functions of these polysaccharides reflect their binding to proteins, through interactions which are largely ionic and involve the negatively charged carboxyl and sulfate groups along with the basic amino acid residues. Sulfation may occur at the *N*-, 3-*O*-, and 6-*O*-positions of glucosamine residues and at the 2-*O*-position of hexuronic acid units (7, 8). The sulfation pattern of HS is tissue-specific (9), varying however with the state of embryonic development (10) and with the age of adult individuals (11). The results of attempts to define in molecular terms the role of heparin/HS in FGF function are intriguing. A minimal saccharide sequence required for FGF-2 binding consists of five monosaccharide units, with *N*-sulfate groups, and a single iduronic acid 2-*O*-sulfate residue but no 6-*O*-sulfate (12–14). By contrast, a sequence of about twice this size is needed to promote FGF-2-induced DNA synthesis (15, 16), which is in accord with proposals that di- or multimerization of FGF-2 molecules along a polysaccharide chain is a prerequisite for receptor dimerization and activation (2, 17, 18). However, analysis of the role of saccharide sulfation suggests that not only 2-*O*- but also one or more 6-*O*-sulfate groups are needed for FGF-2-induced mitogenic signaling (15, 16), potentially implicating binding partners distinct from FGF-2. In fact, hepa-

* This work was supported by grants from Polysackaridforskning AB and from the Medical Research Council project no. K98-03X-12552-01A (to L. C. W.), in part from project no. K99-03X-13004-01A (to M. S.) and K98-03X-2309 (to U. L.), and from the Kung Gustav V Jubileé fond (to L. L. and L. C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 46 18 471 43 63; Fax: 46 18 471 49 75; E-mail: Lena.Welsh@genpat.uu.se.

¹ The abbreviations used are: FGF, fibroblast growth factor; Erk, extracellular signal-regulated kinase; HS, heparan sulfate; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; MBP, myelin basic protein; CAM, chorioallantoic membrane; FGFR, FGF receptor.

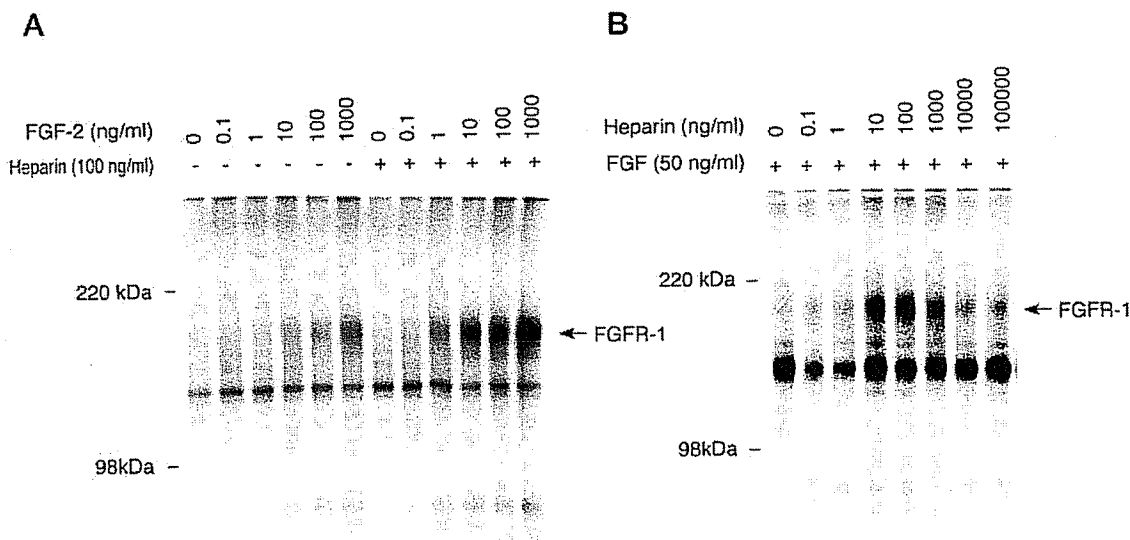


FIG. 1. Dose-dependent amplification by heparin of FGF-2-induced FGFR-1 kinase activity in HS-deficient CHO677 cells. A, CHO677 cells overexpressing FGFR-1 were treated with different concentrations of FGF-2 as indicated, in the absence (–) or presence (+) of 100 ng/ml heparin. B, cells were left untreated or stimulated with 50 ng/ml FGF-1 in the presence of increasing concentrations of heparin. FGFR-1 was immunoprecipitated and analyzed for kinase activity by immune complex kinase assay in the presence of [γ - 32 P]ATP. The migration position of FGFR-1 (140 kDa) is indicated by an arrow to the right. The migration positions of molecular weight markers are indicated on the left.

rin/HS has been shown to interact not only with FGFs but also with their receptors (19–21).

In the present study, we have aimed to define the effects of chain length and specific *O*-sulfate groups on heparin/HS-dependent, FGF-2-induced cellular responses at different stages along the signaling chain. In particular, selectively 2-*O*- and 6-*O*-desulfated heparin derivatives were applied as inhibitors of such reactions, including phosphorylation of FGFR-1 and the mitogen-activated protein kinase Erk2. Moreover, the approach was extended to include FGF-2-induced angiogenesis in the chick chorioallantoic membrane. All of these processes were similarly affected by the desulfated heparins; the 6-*O*-desulfated preparation was inhibitory, whereas the 2-*O*-sulfated preparation was not.

MATERIALS AND METHODS

Cell Culture and Transfection.—The heparan sulfate-deficient Chinese hamster ovary (CHO) cell line 677 (CHO677) (22) was cultured in Ham's F12 medium (Life Technologies, Inc.) supplemented with 7.5% fetal bovine serum (Life Technologies, Inc.), at 37 °C and 5% CO₂. Human FGFR-1 cDNA (23) was subcloned into the pAlter vector (Promega Corporation) and inserted into the eukaryotic expression vector pcDNA1/neo (Invitrogen). CHO677 cells were transfected with the FGFR-1 construct using electroporation; selection of transfected clones was initiated after 48 h by adding geneticin (G418 sulfate, Life Technologies, Inc.). Clones were picked after two weeks and examined for receptor expression by metabolic labeling of the cells with [35 S]methionine.

Immunoprecipitation and In Vitro Kinase Assays.—Confluent cell cultures in 25-cm² flasks (about 1×10^6 cells/flask) were serum-starved for 12 h in Ham's F12 medium supplemented with 0.2% fetal bovine serum. After 7 min of treatment with FGF-2 (FarmItalia Carlo Erba) in the absence or presence of various polysaccharides, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in Nonidet P40 (Nonidet P-40) lysis buffer (1% Nonidet P-40, 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM Na₂VO₄, 1% Trasylol (Bayer), and 1 mM phenylmethylsulfonyl fluoride). Clarified supernatants were incubated with FGFR-1 or Erk2 antisera for 1 h at 4 °C, followed by incubation for 30 min with 40 μ l/ml immobilized protein A (EC Diagnostic, Uppsala, Sweden). The FGFR-1 antiserum was raised against the 16 C-terminal amino acid residues in the human FGFR-1 (23). The Erk2 antiserum was raised in rabbits against a synthetic peptide (EETARFQPGYRS) and was a kind gift from Dr. Lars Rönnstrand, Ludwig Institute for Cancer Research, Uppsala Branch, Sweden. Immobilized immune complexes were washed three times with lysis buffer. Immunocomplexes

were resuspended in 40 μ l of kinase buffer (20 mM Hepes, pH 7.5, 2 mM MnCl₂, 10 mM MgCl₂, 0.05% Triton X-100, and 1 mM dithiothreitol). *In vitro* phosphorylation was carried out by the addition of 5 μ Ci of [γ - 32 P]ATP and incubation for 10 min, at room temperature. The kinase reaction was stopped by the addition of 40 μ l of sample buffer (8% SDS, 0.4 M Tris-HCl, pH 8.0, 1 M sucrose, 10 mM EDTA, 0.02% bromophenol blue, 25 mM dithiothreitol). Samples were boiled for 5 min and resolved by electrophoresis in a 10% SDS-polyacrylamide gel. After fixation in 10% glutaraldehyde, the gel was treated with 1 M KOH for 45 min at 55 °C to hydrolyze serine phosphorylation, dried, and exposed using a Bioimager FUJI BAS-2500. Intensity calculations were made using accompanying software. Rinsed Erk2 immunocomplexes were similarly resuspended in 40 μ l of kinase buffer (20 mM Hepes, pH 8.0, 2 mM MnCl₂, 10 mM MgCl₂, 0.05% Triton X-100, and 1 mM dithiothreitol). Ten μ g/sample myelin basic protein (MBP, Sigma) was included as an exogenous substrate in the kinase reaction. Phosphorylation reactions were carried out by the addition of 5 μ Ci of [γ - 32 P]ATP and incubation for 15 min at room temperature. Samples were processed and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

Glycosaminoglycan Preparations.—Heparin from pig intestinal mucosa was purified as described previously (24). Heparan sulfate from bovine intestine was a generous gift of Dr. Keiichi Yoshida, Seikagaku Corporation, Tokyo, Japan. Chondroitin sulfate A was from Sigma. The preparations of *N*-, 2-*O*-, and 6-*O*-desulfated heparins and heparin oligosaccharides were as described earlier (25, 26). In the 2-*O*-desulfated heparin/heparin oligosaccharides, ~1% of the iduronic acid residues were 2-*O*-sulfated, whereas >80% of the *N*-sulfoglucosamine residues were 6-*O*-sulfated. In the 6-*O*-desulfated preparations, the degree of glucosamine 6-*O*-sulfation was <10%, but the treatment also resulted in the removal of ~30% of the 2-*O*-sulfate groups. The preparations were subjected to high resolution gel filtration and were sterile-filtered to remove possible contamination. There was no sign of toxicity in tissue culture.

[3 H]Thymidine Incorporation Assay.—Parental CHO677 cells were grown to 50% confluency in 12-well plates (about 100,000 cells/well). Medium was replaced with serum-free medium containing 0.2% bovine serum albumin, and the cultures were incubated for 48 h. At this point, fresh serum-free medium containing FGF-2 alone or in combination with the different heparin preparations, as described in the figure, legend were added. [3 H]Thymidine (Amersham Pharmacia Biotech, 0.5 μ Ci/well) was added 4 h before harvesting. Cells were then treated with ice-cold 5% trichloroacetic acid for 20 min, washed three times with ice-cold water, and then solubilized with 1 M NaOH, and 3 H radioactivity was determined by liquid scintillation counting.

Peptide Synthesis and Affinity Chromatography.—The peptide KMEKKLHVPAAKTVKFK-COOH (K18K) corresponding to amino acid residues Lys¹⁶⁹ to Lys¹⁷⁷ in the human FGFR-1 (according to Swiss

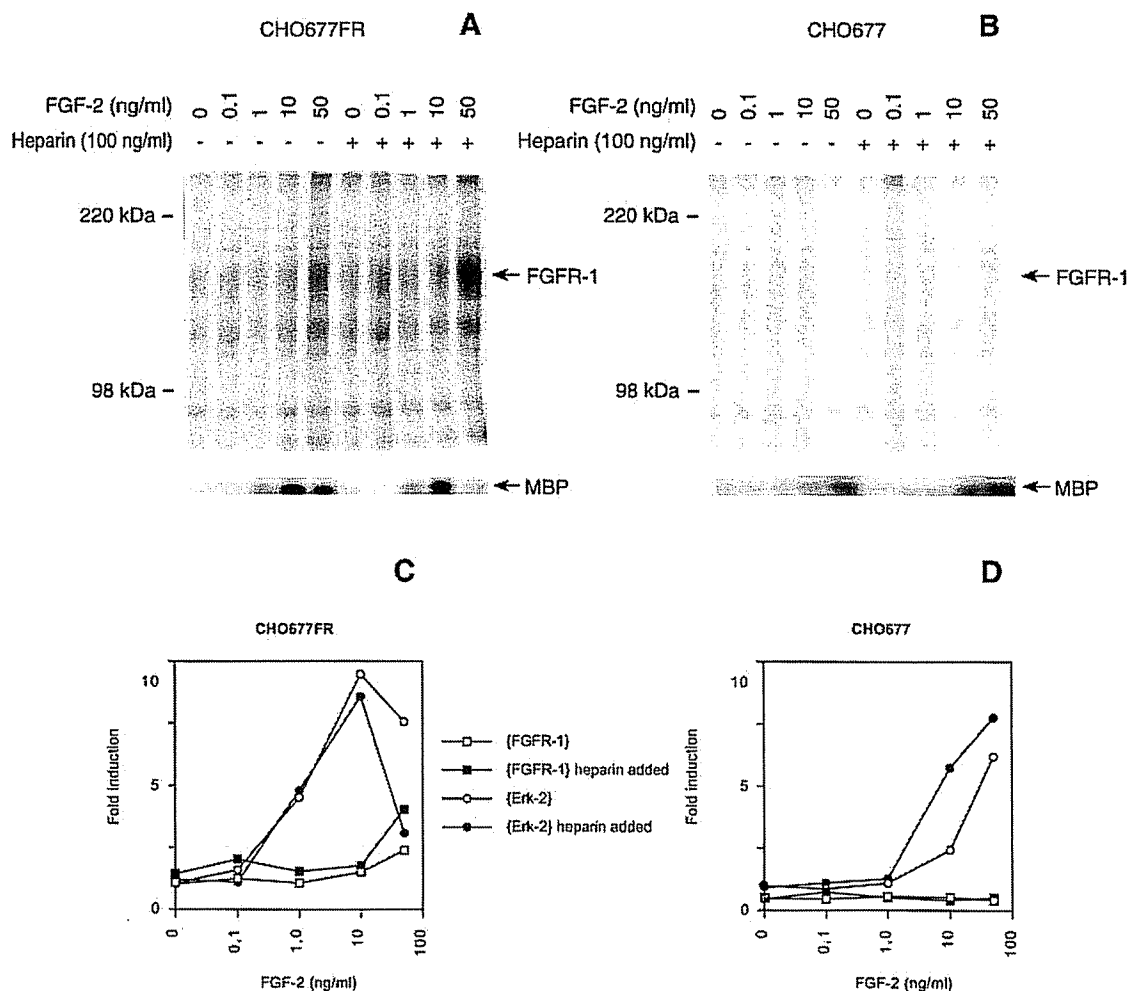


FIG. 2. Effect of heparin on FGF-2-induction of FGFR-1 and Erk2 kinase activities in FGFR-1 overexpressing CHO677 and parental cells. A, CHO677 cells overexpressing FGFR-1 were stimulated with increasing concentrations of FGF-2 with or without 100 ng/ml heparin. Lysates were assayed for kinase activity of immunoprecipitated FGFR-1 (upper panel) and Erk2 (lower panel), in the latter case using MBP as an exogenous substrate. The migration positions of FGFR-1 and MBP are indicated, as well as migration positions of molecular weight markers. B, parental CHO677 cells were stimulated and assayed as in A. C and D, quantification of FGFR-1 and Erk2 activation in response to FGF-2 and heparin in the two cell types.

Prot accession number p11362), was synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry, as described by Mori *et al.* (27). A cysteine residue was added to the N terminus of the peptide to allow coupling to SulfoLink[®] coupling gel (Pierce). Coupling was performed using 0.4- μ mol peptide and 2 ml of SulfoLink[®] gel. The resulting column was equilibrated with PBS containing 3 mM CaCl₂. Samples of ³H-labeled native, 2-*O*-desulfated or 6-*O*-desulfated 18-mer oligosaccharides were applied in 0.5 ml of the same buffer. The bound saccharides were eluted using a linear gradient of NaCl (0–1.2 M) in 15 mM Tris-HCl, pH 7.4, 3 mM Ca²⁺. Fractions (1 ml) were collected and analyzed for radioactivity and ionic strength by liquid scintillation counting and conductometry, respectively.

The FGF-2 affinity column was prepared by mixing 1 mg of recombinant FGF-2 with a 2-fold molar excess of heparin, followed by immobilization of FGF to 1.5 ml of activated CH-Sepharose 4B (Amersham Pharmacia Biotech). To prevent binding of heparin to the column, the heparin preparation had previously been treated with HNO₂ at pH 3.9, thereby eliminating *N*-unsubstituted GlcN units, with concomitant chain cleavage, followed by recovery of high molecular weight species by gel filtration. The FGF-2-Sepharose was transferred to a column and washed extensively with 2 M NaCl and then equilibrated in 50 mM Tris-HCl, pH 7.4. Radiolabeled saccharides were eluted using a linear gradient of NaCl (0–2 M) in 50 mM Tris-HCl, pH 7.4. Fractions (1 ml) were collected and analyzed for radioactivity and ionic strength by liquid scintillation counting and conductometry, respectively.

Chorioallantoic Membrane Angiogenesis Assay—The conditions for the chorioallantoic membrane (CAM) assay essentially followed a pre-

viously described procedure (28, 29). Fertilized chick eggs were purchased locally and preincubated for ten days at 38 °C at 70% humidity. The CAM was exposed by a 1-cm² window in the shell, and an avascular zone was identified for sample application. Whatman filter disks (5 mm in diameter) were saturated with 3 mg/ml cortisone acetate (Sigma) and soaked in 40 μ l of PBS containing 0.2 μ g of FGF-2 with or without 6-*O*- or 2-*O*-desulfated heparin. The window was sealed with tape and incubated for three additional days. The CAM was then cut around the filter and examined using a Nikon Eclipse TE 300 light microscope (magnification 2.5 or 4). Angiogenesis was scored in a double blind procedure for each embryo by estimating the number of vessel branch points in the membrane on the filter disc. The scores ranged from 1 (low, PBS background) to 4 (high). Each substance was analyzed in parallel with 5 to 7 embryos. Sample variation was less than 15%. P values were calculated with ANOVA (analysis of variance).

RESULTS

Dose-dependent Amplification of FGF-2-induced FGFR-1 Activation by Heparin in HS-deficient CHO Cells—Heparin is known to be critical for induction of DNA synthesis in response to FGF-2 treatment of HS-deficient cell models (6, 12, 15, 16). To delineate the mechanisms underlying this effect, we monitored FGFR-1 kinase activity in the HS-deficient CHO cell line 677 transfected with FGFR-1 (denoted CHO677FR). Cells were treated with increasing concentrations of FGF-2 in the absence

or presence of 100 ng/ml heparin (Fig. 1A) or with increasing concentrations of heparin in the presence of 50 ng/ml FGF-2 (Fig. 1B). After stimulation, cells were lysed and immunoprecipitated with antibodies specific for FGFR-1. The immunoprecipitated receptor was assayed for kinase activity (auto- ^{32}P -phosphorylation) as described under "Materials and Methods." FGF-2 alone induced substantial FGFR-1 kinase activity at a concentration of 1 $\mu\text{g/ml}$ (Fig. 1A). The addition of heparin amplified the effect of FGF-2. Quantification (not shown) of the FGFR-1 intensities in the autoradiogram in Fig. 1A indicated that, at 100 ng/ml, heparin-mediated amplification was maximal, three times greater than that of FGF-2 alone, at a concentration of 1 ng/ml FGF-2, and somewhat less at higher FGF-2 concentrations. Fig. 1B shows that incorporation of ^{32}P in FGF-2-stimulated FGFR-1 increased with increasing concentrations of heparin up to 100 ng/ml; higher concentrations of heparin reduced the effect, probably because of dilution of the FGF-2 pool. Heparin alone at concentrations between 1 ng/ml and 100 $\mu\text{g/ml}$ had no effect on FGFR-1 kinase activity (data not shown). These data show that heparin/Hs is required for FGF-2 induction of receptor kinase activity.

Function of Heparin in Signal Transduction Downstream of FGFR-1—The serine kinases Erk1 and -2 are known to be involved in signal transduction downstream of FGFR-1 and are critical for the proliferative response to growth factors (30). We analyzed the effects of heparin on FGF-2-induced Erk2 kinase activity. Cells were stimulated with various concentrations of FGF-2 in the presence or absence of heparin. In CHO677 cells overexpressing FGFR-1, receptor kinase activity was induced in a dose-dependent manner by FGF-2 and was potentiated by heparin, as shown above. Erk kinase activity, estimated by the extent of phosphorylation of the exogenous substrate MBP, peaked at 10 ng/ml FGF-2 both in the absence and presence of heparin (Fig. 2, A and C). At higher concentrations of FGF-2, Erk2 kinase activity decreased, especially in the presence of heparin. We then employed parental CHO677 cells to further examine the effect of heparin on Erk kinase activity. In the parental cells, FGFR-1 phosphorylation could not be detected by *in vitro* kinase assays because of the low level of receptor expression (Fig. 2B). There are about 1000 FGF receptor molecules/cell on the CHO677 cells.² Some or all of these FGF receptormolecules correspond to FGFR-1, as indicated by polymerase chain reactions using FGFR-1-specific primers (data now shown). Stimulation of these endogenous FGF receptors with FGF-2 led to induction of Erk2 kinase activity in a dose-dependent manner, and inclusion of heparin potentiated the effect (Fig. 2, B and D). Thus, activation of a small pool of receptors, undetectable in the parental CHO677 cells by *in vitro* kinase assay, appears to elicit signal transduction that is amplified by heparin, resulting in detectable activation of Erk2. In cells overexpressing FGFR-1, the input signals at high doses of FGF-2 together with heparin may lead to a compensatory down-regulation of Erk signal transduction. In the following experiments, we have monitored the effects of heparin on FGFR-1 kinase using the FGFR-1-transfected CHO677 cells and effects on Erk2 using the parental CHO677 cells.

Heparin Fragments Longer than 8/10 Monosaccharide Units Amplify FGF-2-induced FGFR-1 and Erk2 Kinase Activity—The smallest fragment of heparin able to rescue the FGF-2-induced increase in DNA synthesis in two different HS-deficient cell lines has been shown to encompass 10–12 monosaccharide units (15, 16). Fig. 3 shows CHO677 cells overexpressing FGFR-1 stimulated with FGF-2 in the presence of heparin fragments of increasing size. Fragments larger than

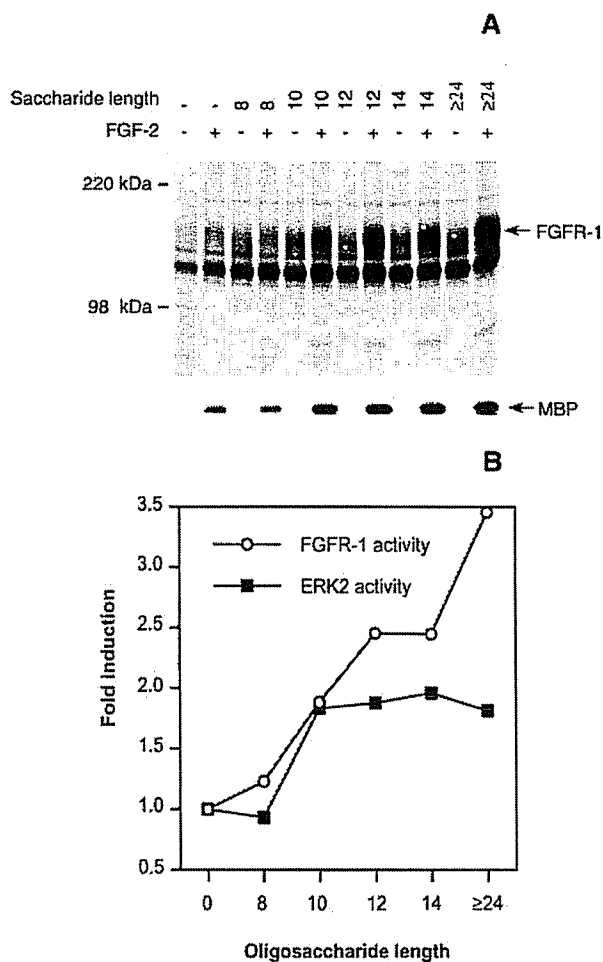


FIG. 3. The heparin chain length influences the efficiency of FGFR-1 kinase activation in response to FGF-2. A, CHO677 cells overexpressing FGFR-1 were stimulated with 50 ng/ml FGF-2 in the absence or presence of 100 ng/ml heparin oligomers of defined length. The upper panel shows immunoprecipitation of FGFR-1 followed by *in vitro* kinase assay as described above. The lower panel shows samples from parental cells, stimulated with 10 ng/ml FGF-2, and assayed for Erk2 kinase activity using MBP as an exogenous substrate. B, BioImager quantification of FGFR-1 (circles) and Erk2 (squares) kinase activities.

8/10 monosaccharide units stimulated FGFR-1 markedly better than shorter heparin fragments (see Fig. 3B for quantification). A similar pattern was seen when analyzing induction of Erk2 kinase activity in parental CHO677 cells (Fig. 3, lower panel). Treatment with longer heparin fragments led to a further increase in FGFR-1 kinase activity, whereas Erk2 kinase activity remained at the same level with increasing length of the heparin chain.

Sulfation at 2-O- and 6-O-Positions in Heparin Is Required for Potentiation of FGFR-1 and Erk2 Kinase Activities—Heparin is sulfated mainly at N- and 6-O-positions of the glucosamine residues and at the 2-O-position of iduronic acid units (7). Binding of heparin to FGF-2 has been shown to require sulfation only at the N- and 2-O-positions (13, 14, 31). This binding was confirmed using affinity chromatography of selectively desulfated heparin preparations on immobilized FGF-2. 2-O-Desulfated heparin showed considerably reduced binding to FGF-2 (elution at 0.35 M NaCl), whereas 6-O-desulfated heparin bound essentially with the same efficiency as intact heparin (elution at 1.5 M NaCl, Fig. 4A). The desulfated heparin preparations were further tested for their abilities to support FGF-

² A. Yayon, personal communication.

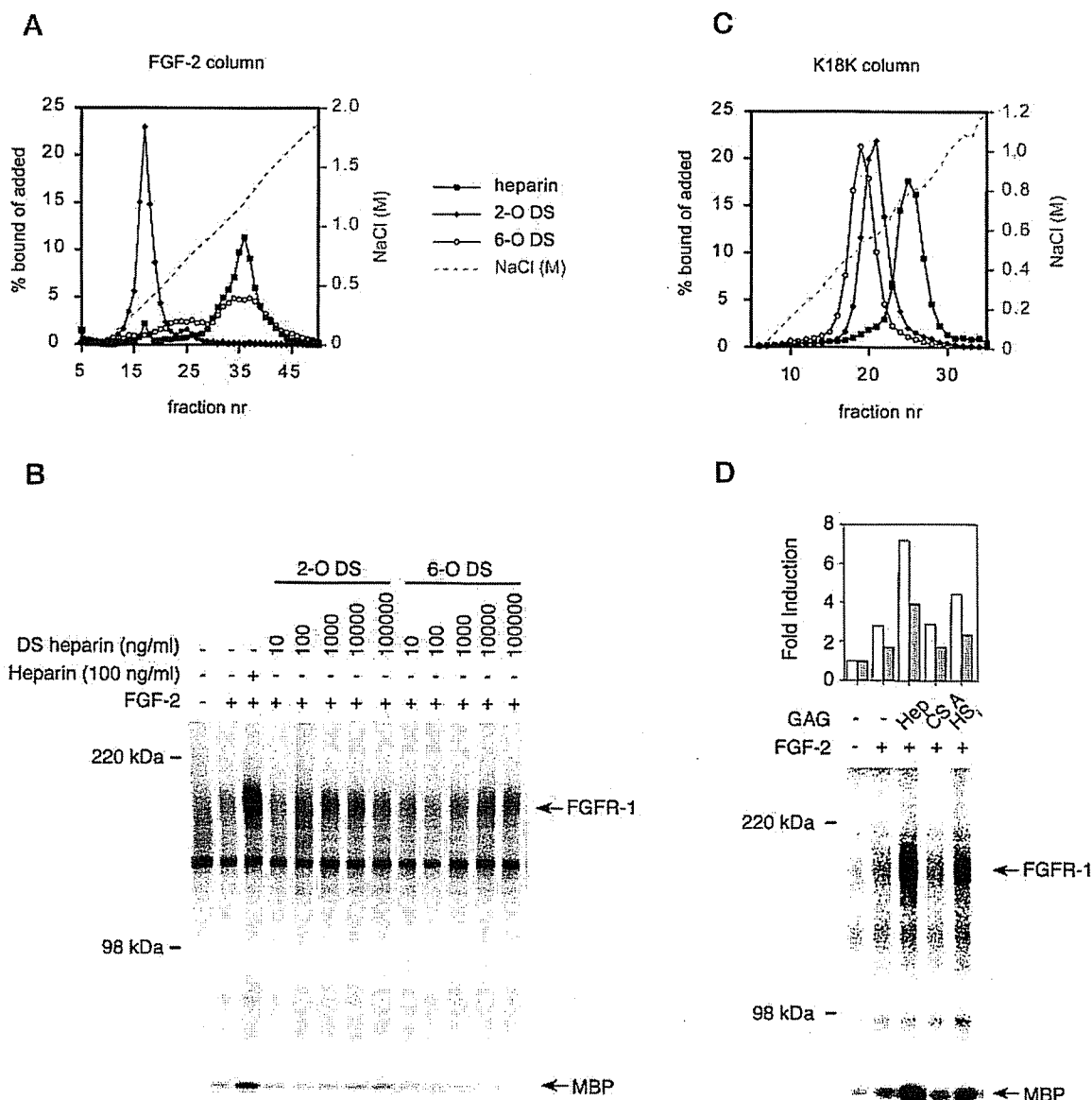


Fig. 4. Sulfation requirement of HS/heparin for FGFR-1 signal transduction. **A**, ^3H -labeled oligosaccharides prepared from intact heparin; 2-*O*- and 6-*O*-desulfated (DS) heparin were analyzed for binding to immobilized FGF-2. Bound material was eluted using a linear NaCl gradient. **B**, induction of FGFR-1 and Erk2 kinase activities in CHO677 cells overexpressing FGFR-1, treated with FGF-2 in the absence and presence of different concentrations of unlabeled native, 2-*O*-desulfated (2-*O* DS) or 6-*O*-desulfated (6-*O* DS) heparin. FGFR-1 and Erk2 were immunoprecipitated, and the samples were subjected to *in vitro* kinase assay as described in the legend to Fig. 3. **C**, affinity chromatography of ^3H -labeled 18-mer oligosaccharide of intact heparin, 6-*O*-, and 2-*O*-desulfated heparin on an immobilized K18K peptide (corresponding to a portion of the extracellular domain of FGFR-1). Bound material was eluted using increasing concentrations of NaCl. **D**, FGFR-1 overexpressing and parental CHO677 cells were left untreated, stimulated with FGF-2 alone, or in combination with native heparin, chondroitin sulfate, or HS from bovine intestine, as indicated. FGFR-1 and Erk2 kinase activities were assayed as described above. The upper panel shows quantification of the ^{32}P incorporation in the FGFR-1 and Erk2 bands (open and filled bars, respectively). GAG, glycosaminoglycan.

2-*O*-induced FGFR-1 and Erk2 kinase activities (Fig. 4B). As seen in Fig. 4B, neither the 2-*O*- nor the 6-*O*-desulfated heparin preparations were able to support FGFR-1 and Erk2 kinase activities. These data agree with previous findings that sulfation at both the 2-*O*- and 6-*O*-positions is required for FGF-2-induced DNA synthesis (15, 16), and hence with the notion that the latter sulfate substituent may be implicated in receptor binding. An 18-amino acid-residue sequence (KMEKKLHAVPAAKTVKFK) has been identified as a potential heparin binding region in the FGFR-1 extracellular domain (20). This stretch was synthesized and immobilized. The desulfated heparin preparations were applied on the column and eluted using a NaCl gradient (Fig. 4C). Both the 6-*O*- and the

2-*O*-desulfated heparin bound considerably less strongly to the peptide than intact heparin. Together, our data support a model where 2-*O*-sulfation of heparin mediates binding to FGF-2, whereas both 6-*O*- and 2-*O*-sulfation are required for binding to the receptor. Thus, formation of a ternary complex including FGF-2, heparin, and FGFR-1 appears to be required for activation of the FGFR-1 kinase.

Ability of Heparan and Chondroitin Sulfate to Amplify FGF-2-induced FGFR-1 and Erk2 Kinase Activities—Specific sulfation patterns determine the effects of HS in biological processes such as coagulation (32), FGF-2-induced DNA synthesis (15, 16), and adhesion of proteoglycans to extracellular matrix components (33). We examined the effects of bovine intestine-

derived HS on FGF-2-stimulated FGFR-1 overexpressing and parental CHO677 cells, in comparison with heparin and chondroitin sulfate (Fig. 4D). Heparin was most efficient in supporting induction of FGFR-1 kinase activity (3-fold increase as compared with FGF-2 alone). In cells treated with intestinal HS, FGFR-1 kinase activity was doubled, whereas chondroitin sulfate showed no amplifying effect.

6-O-Desulfated Heparin Is a Potent Inhibitor of Heparin-amplified, FGF-2-induced FGFR-1 and Erk2 Kinase Activities and Subsequent Mitogenicity—Because 6-O-desulfated heparin binds to FGF-2 but only weakly to the receptor (see Fig. 4, A and C), it may block formation of the ternary FGF-2-heparin-FGFR-1 complex. Accordingly, addition of a 10-fold excess of 6-O-desulfated heparin over intact heparin completely abolished the amplifying effect of heparin on FGF-2-induced FGFR-1 and Erk2 kinase activities (Fig. 5A). In contrast, 2-O-desulfated heparin, which does not bind to FGF-2, failed to compete with intact heparin. Moreover, DNA synthesis in CHO677 cells treated with FGF-2 was increased in the presence of heparin and this effect was blocked when 6-O-desulfated heparin was included together with FGF-2 and intact heparin (Fig. 5B). In contrast, 2-O-desulfated heparin showed no inhibitory effect.

6-O-Desulfated Heparin Inhibits FGF-2-induced Angiogenesis in the CAM Assay—FGF has been shown to promote endothelial cell responses *in vitro* and angiogenesis *in vivo* (34). We tested the potential of 6-O-desulfated heparin to inhibit FGF-2-induced angiogenesis in day 10 chicken embryos. Angiogenesis was efficiently induced by FGF-2 alone (Fig. 6). Co-incubation of FGF-2 and a 10-fold molar excess of 2-O-desulfated heparin did not perturb the formation of blood vessels. In contrast, a 10-fold molar excess of 6-O-desulfated heparin led to a significant reduction ($p < 0.01$) in vessel diameter of newly formed vessels, whereas preformed vessels were unaffected. Additionally the 6-O-desulfated heparin affected the continuity of the scarce newly formed vessels, which appeared constricted at regular intervals (Fig. 6D). 6-O-Desulfated heparin thus acts as an angiogenesis inhibitor in the CAM, leading to thin and discontinuous vessels, whereas 2-O-desulfated heparin has no effect on FGF-2-induced angiogenesis.

DISCUSSION

The requirement for heparin/HS in FGF-2-induced DNA synthesis or cell differentiation has been established using different HS-deficient cell models. It has been shown that FGF-2 fails to repress differentiation of chlorate-treated MM14 myoblasts (6). There is furthermore no increased [3 H]thymidine incorporation in FGF-2-treated BAF32 cells (16), which are devoid of endogenous HS, nor in chlorate-treated NIH3T3 mouse fibroblasts (6, 15). We have employed the HS-deficient CHO677 cell line to analyze in greater detail the sulfate dependence of FGF-2-induced cellular responses.

Two strategies have been pursued. First, selectively O-desulfated heparins have been tested for their ability to promote effects of FGF-2 in the HS-deficient cells. Second, the same modified heparins have been assessed for their ability to inhibit FGF-2 effects that are promoted by fully sulfated heparin and by endogenous HS. By these approaches we could demonstrate that FGF-2-induced FGFR-1 activation, Erk2 kinase induction, and angiogenesis all depend on a saccharide containing both 2-O- and 6-O-sulfate groups. Because binding of FGF-2 to heparin/HS requires 2-O-sulfate only (13, 14, 31), it seems reasonable to conclude that the 6-O-sulfation is required for another interaction.

This suggestion may be evaluated in relation to various current models of heparin/HS-supported FGF-2 action. It has been proposed that the role of the saccharide is to present FGF

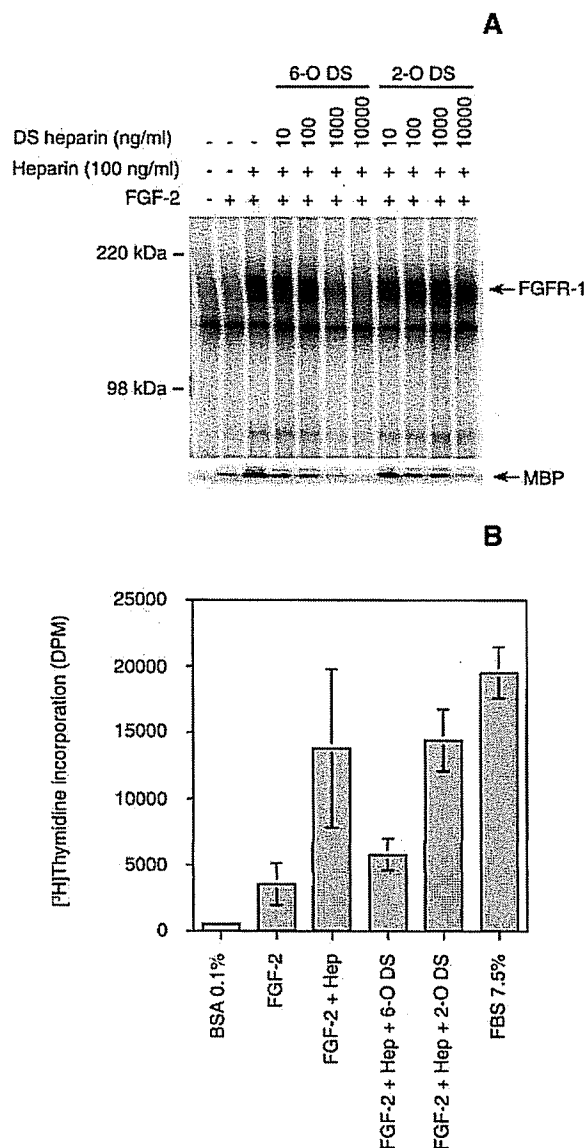


FIG. 5. 6-O-Desulfated heparin is a potent inhibitor of FGFR-1 and Erk2 kinase activities and DNA synthesis. A, CHO677 cells overexpressing FGFR-1 were treated with and without FGF-2 (50 ng/ml) and heparin (100 ng/ml) in the absence or presence of different concentrations of 6-O- (6-O DS) or 2-O- (2-O DS) desulfated heparin (upper part). In parallel, parental CHO677 cells were treated with FGF-2 (10 ng/ml) and heparin (100 ng/ml) in the presence or absence of selectively desulfated heparin (lower part). The kinase activities of immunoprecipitated FGFR-1 (upper part) and Erk2 (lower part) were examined by *in vitro* kinase assays as described above. The migration positions of FGFR-1, MBP (exogenous substrate for Erk2) and marker proteins are indicated. B, parental CHO677 cells were grown in serum-free medium for 48 h followed by incubation for 24 h with 10 ng/ml FGF-2 in the presence or absence of 100 ng/ml heparin. Indicated cultures also received a 10-fold excess of 2-O- or 6-O-desulfated heparin (denoted 2-O DS and 6-O DS, respectively). [3 H]Thymidine at 0.5 μ Ci/ml was added 4 h before harvesting. Cells treated with 7.5% of serum served as positive control. The results show mean \pm S.E. of three parallel wells.

to its tyrosine kinase receptors in a dimeric fashion, thereby facilitating dimerization of receptors (2). The high affinity binding of FGF-2 to heparin fits with this model. Activation of receptor tyrosine kinases is critically dependent on the formation of receptor dimers (35), which could be facilitated by dimerization of the monomeric FGF. Indeed, dimerization of platelet-derived growth factor is dependent on the dimeric con-



FIG. 6. **6-O-Desulfated heparin inhibits FGF-2-induced angiogenesis in the chicken CAM assay.** The CAM of 10-day-old chick embryos was exposed, and filter discs soaked in PBS, in 2 μ g of FGF-2 alone, or in FGF-2 in combination with a 10-fold excess of 2-O-desulfated or 6-O-desulfated heparin was placed on the CAM. The windows were sealed with tape and incubated for three more days and examined under a microscope.

figuration of the ligand. However, this model does not readily explain the requirement for 6-O-sulfate groups in promoting FGF-induced effects. Conceivably, this residue could be involved in the binding of a second FGF-2 molecule, on the opposite side of the saccharide backbone relative to an FGF-2 binding site involving a 2-O-sulfate group only (17). On the other hand, a number of observations suggest involvement of the receptor in heparin/HS binding, where the saccharide bridges the growth factor and the receptor molecules (19, 20, 36). Kan *et al.* (20) identified a heparin binding peptide (denoted K18K) in the extracellular domain of FGFR-1 and showed that this peptide is able to block FGF-induced cellular responses. Our present results demonstrate that binding of heparin to this peptide is dependent on 6-O- as well as 2-O-sulfation of the saccharide. A requirement for 2-O-sulfation for FGF-2 binding and 2-O- and 6-O-sulfation for receptor binding would be in excellent agreement with the finding that the minimal HS oligosaccharide able to rescue FGF-2-induced biological responses in HS-deficient cells is a 10/12-mer containing both 2-O- and 6-O-sulfate groups (15, 16). A further argument in favor of the bridging hypothesis rather than the FGF dimerization model, is the finding by Pye and Gallagher (37) that a conjugate of an FGF-2 monomer covalently bound to a heparin oligosaccharide showed mitogenic activity. Taken together, the available evidence would tend to favor the involvement of a ternary complex involving a HS sequence that binds to both FGF and to the receptor. The detailed composition of such as complex remains to be determined. Recent crystallographic data identify complexes composed of two sets of FGF ligand-receptor extracellular domain units. The units are pictured by molecular modeling to be held together by a polysaccharide string that traverses from one FGF molecule through a groove between the two receptor molecules, containing the heparin binding receptor sequence identified by Kan *et al.* (20), to finally reach the second FGF molecule (38).

Our data furthermore suggest that heparin does not contribute solely by increasing the number of active, signaling receptors but possibly also by increasing the number of phosphorylation sites available for the kinase, resulting in a more efficient phosphorylation of individual receptors. This argument is based on data showing that heparin increases the affinity of FGF-2 in binding to FGFR-1, thereby decreasing the dissociation constant from 40 to 15 pM (39). One μ g of FGF-2 (47.8 nM)/ml, as added in Fig. 1A, corresponds to a concentration 1000-fold above the K_d value and should saturate the cell surface expressed FGF receptors with or without heparin. However, there is still a strong increase in FGFR-1 autophosphorylation when heparin is added in combination with FGF-2 at 1 μ g/ml. Our preliminary data using two-dimensional separation of FGFR-1 tryptic phosphopeptides indicate that heparin increases the utilization of certain phosphorylation sites more than others, compatible with a change in FGFR-1 confor-

mation in the presence of heparin.³ FGF-2 alone to some extent stimulated FGFR-1 and Erk2 kinase activities in CHO677 cells (Fig. 1A). A similar level of Erk2 activation by FGF-2 alone was recorded in CHO745 cells (22), which lack expression of both heparan sulfate and chondroitin sulfate (data now shown). These data indicate that heparin modulates, but is not strictly required for, all FGF-2 effects.

There is a clear specificity in heparin sulfation required for biological activity of FGF (15, 16). The fact that sulfation is organ specific (9) suggests that the effect of FGF *in vivo* does not only depend on expression of FGF and FGF receptors, but also on the activating HS structures (see Fig. 4D). We show that 6-O-desulfated heparin may block FGF-2-induced biological effects by competing with endogenous heparan sulfate and efficiently inhibits angiogenesis in the chicken CAM. Deregulated angiogenesis is recognized as a complicating factor in a wide spectrum of diseases, notably in tumor progression. The potential of anti-angiogenic treatment, *e.g.* for vascularized solid tumors, is currently intensely explored. Treatment using desulfated heparin would inhibit the FGF-induced effects in the disease, and it may also affect other factors such as vascular endothelial growth factor, which is a heparin binding growth factor acting on endothelial cells (40). Our future efforts will be aimed at further exploring the potential of heparin derivatives in treatment of FGF- and vascular endothelial growth factor-induced angiogenesis.

Acknowledgments—We thank Dr. Jeffery D. Esko, Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, for providing us with the HS-deficient CHO677 cells; Drs. Dorothe Spillmann and Camilla Westling, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden, for generous sharing of heparin preparations; and Dr. K. Yoshida, Seikagaku Corporation, Tokyo, Japan, for bovine HS preparation.

REFERENCES

1. Klint, P., and Claesson-Welsh, L. (1999) *Front. Biosci.* 4, 165–177
2. Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) *Cell* 79, 1015–1024
3. Wiedlocha, A., Farnes, P. O., Madhus, I. H., Sandvig, K., and Olsnes, S. (1994) *Cell* 76, 1039–1051
4. Klagsbrun, M. (1992) *Semin. Cancer Biol.* 3, 81–87
5. Kato, M., Wang, H., Kainulainen, V., Fitzgerald, M. L., Ledbetter, S., Ornitz, D. M., and Bernfield, M. (1998) *Nat. Med.* 4, 691–697
6. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) *Science* 252, 1705–1708
7. Salmivirta, M., Lidholt, K., and Lindahl, U. (1996) *FASEB J.* 10, 1270–1279
8. Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) *J. Biol. Chem.* 273, 24979–24982
9. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem.* 271, 17804–17810
10. Brickman, Y. G., Ford, M. D., Gallagher, J. T., Nurcombe, V., Bartlett, P. F., and Turnbull, J. E. (1998) *J. Biol. Chem.* 273, 4350–4359
11. Feyzi, E., Saldeen, T., Larsson, E., Lindahl, U., and Salmivirta, M. (1998) *J. Biol. Chem.* 273, 13395–13398
12. Ishihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S., Cousens, L. S., and Stack, R. J. (1993) *J. Biol. Chem.* 268, 4675–4683

³ L. Lundin and L. Claesson-Welsh, manuscript in preparation.

13. Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem.* **268**, 23898–23905
14. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) *Science* **271**, 1116–1120
15. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268**, 23906–23914
16. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) *J. Biol. Chem.* **273**, 22936–22942
17. DiGabriele, A. D., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J., and Hendrickson, W. A. (1998) *Nature* **393**, 812–817
18. Moy, F. J., Safran, M., Seddon, A. P., Kitchen, D., Bohlen, P., Aviezer, D., Yayon, A., and Powers, R. (1997) *Biochemistry* **36**, 4782–4791
19. Kinsella, L., Chen, H. L., Smith, J. A., Rudland, P. S., and Fernig, D. G. (1998) *Glycoconj. J.* **15**, 419–422
20. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) *Science* **259**, 1918–1921
21. Gao, G., and Goldfarb, M. (1995) *EMBO J.* **14**, 2183–2190
22. Esko, J. D. (1991) *Curr. Opin. Cell Biol.* **3**, 805–816
23. Wennstrom, S., Sandstrom, C., and Claesson-Welsh, L. (1991) *Growth Factors* **4**, 197–208
24. Lindahl, U., Cifonelli, J. A., Lindahl, B., and Rodén, L. (1965) *J. Biol. Chem.* **240**, 2817–2820
25. Feyzi, E., Lustig, F., Fager, G., Spillmann, D., Lindahl, U., and Salmivirta, M. (1997) *J. Biol. Chem.* **272**, 5518–5524
26. Feyzi, E., Trybala, E., Bergstrom, T., Lindahl, U., and Spillmann, D. (1997) *J. Biol. Chem.* **272**, 24850–24857
27. Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtneidge, S. A., Claesson-Welsh, L., and Heldin, C. H. (1993) *EMBO J.* **12**, 2257–2264
28. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresch, D. A. (1995) *Science* **270**, 1500–1502
29. Brooks, P. C., Clark, R. A., and Cheresch, D. A. (1994) *Science* **264**, 569–571
30. Klint, P., Kanda, S., Kloog, Y., and Claesson-Welsh, L. (1999) *Oncogene* **18**, 3354–3364
31. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) *J. Biol. Chem.* **267**, 10337–10341
32. Rosenberg, R. D. (1989) *Am. J. Med.* **87**, 2S–9S
33. Sanderson, R. D., Turnbull, J. E., Gallagher, J. T., and Lander, A. D. (1994) *J. Biol. Chem.* **269**, 13100–13106
34. Klein, S., Roghani, M., and Rifkin, D. B. (1997) *Exs* **79**, 159–192
35. Heldin, C. H. (1995) *Cell* **80**, 213–223
36. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry* **33**, 10229–10248
37. Pye, D. A., and Gallagher, J. T. (1999) *J. Biol. Chem.* **274**, 13456–13461
38. Plotnikov, A. N., Schlessinger, J., Hubbard, S. R., and Mohammadi, M. (1999) *Cell* **98**, 641–650
39. Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rifkin, D. B., and Moscatelli, D. (1994) *J. Biol. Chem.* **269**, 3976–3984
40. Ferrara, N. (1999) in *Vascular Growth Factors and Angiogenesis in Current Topics in Microbiology and Immunology* (Claesson-Welsh, L., ed) Vol. 237, pp. 1–30, Springer-Verlag, Berlin